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Enhancement of RNA Polymerase II Initiation Complexes by a Novel DNA Control Domain Downstream from the Cap Site of the Cytomegalovirus Major Immediate-Early Promoter†

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The major immediate-early promoter (MIEP) of human cytomegalovirus is a remarkably strong RNA polymerase II transcription control unit. We have identified and characterized a novel regulatory domain associated with MIEP downstream from the initiation site of transcription. The downstream regulatory region was first identified by analyzing a series of mutations in the 5' untranslated leader exon. This regulatory domain was shown to enhance the number of functional initiation complexes without significantly altering the apparent elongation rate by RNA polymerase II transcription. In addition, run-off *in vitro* transcription and DNA-binding experiments identified two distinct downstream elements that specify the interaction of cellular transcription factors. One of these elements contains a reiterated sequence motif, present twice within the leader exon. The second element is an 18-bp sequence located at approximately nucleotide position +33 that is conserved between strains of cytomegalovirus from different species. On the basis of two criteria, an oligonucleotide competition assay and oligomerization upstream of the promoter, the binding of factors to the conserved box was shown to be critical for mediating the level of transcription from MIEP. Two discrete cellular nuclear proteins, designated LTF A and B (for leader transcription factor A and B binding factors), were found to specifically recognize the conserved element. This study of promoter-proximal elements within transcribed sequences demonstrates the recognition of the control domain at the DNA level that functions to increase the number of committed RNA polymerase II transcription complexes.

Regulation of viral immediate-early (IE) gene expression at the level of transcription constitutes a primary level by which persistent viruses may regulate their state of activation in the host. We are interested in understanding the role of cellular and viral transcription factors in coordinating RNA polymerase II activity associated with the UL123-122 (7) major IE promoter (MIEP) of human cytomegalovirus (HCMV), an important human pathogen. Since the MIEP is dependent on the host cell transcriptional apparatus, it also provides a particularly useful tool for detecting features of the mammalian transcription control machinery.

Regulatory sequences of the MIEP offer a spectrum of distinct upstream control domains encompassing an RNA polymerase II promoter between +1 and –50 (17, 54), a strong enhancer between –50 and –550 (3, 16, 52), a unique sequence region (19) adjacent to a cluster of NF1/CTF binding sites between –550 and –750 (19, 24, 28), and a modulator sequence between –750 and –1145 (39, 49). The modulator regulates, cell type specifically, transcription from the MIEP in both a negative and positive manner (33, 39, 49). Conversely, the enhancer contains a complex array of both strong constitutive and inducible regulatory elements (6, 18, 26, 41, 46, 51). Certain members of these regulatory modules contain binding sites for cellular transcription factors that have been characterized in other gene promoter systems (reviewed in reference 20). However, the HCMV enhancer modules also interact with a number of cellular

transcription factors in cellular gene regulation that have yet to be identified (18). Thus, the interfacing of MIEP with a variety of sequence-specific transcription factors provides a means to elucidate the function of a variety of cellular regulatory proteins with relevance to the physiology of both the cell and the virus.

The promoter-proximal downstream sequences of a number of viral and cellular genes transcribed by RNA polymerase II have recently been recognized as containing transcriptional regulatory elements (1, 8, 11, 22, 25, 29, 32, 35, 40, 53, 56, 57). In contrast to upstream regulatory domains, the privileged location of *cis*-acting elements downstream of the cap site provides the ability of *trans*-acting factors to operate at two unique levels. First, these transcriptional elements have the opportunity of being recognized at either the DNA or the RNA level. Second, the level by which they affect transcription can be at the rate of RNA polymerase initiation or elongation. Here, we report the identification and characterization of a novel transcription control domain within the 5' untranslated leader exon of the HCMV UL123 gene. In this study we have attempted to define the molecular requirements of the promoter-proximal downstream elements as well as the levels by which they may interact with the transcriptional machinery.

MATERIALS AND METHODS

Plasmid constructions. The plasmid pUCExon1 contains the *SacI*-*MaeIII* fragment (*MaeIII* end of the fragment was blunted by using mung bean nuclease) from the vector pUCSst0.75, containing MIEP sequences from –19 to +715.

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The *SacI*-*MaeIII* fragment contains MIEP sequences from -19 to +112 and was cloned into the pUC19 vector between the *SacI* and the *HindIII* sites. The recombinant plasmid pMIEP(-1145/+7)CAT contains the *PstI*-*Sau3A* fragment of MIEP fused to the *cat* gene as described previously (26). The construction of pMIEP(-1145/+54)CAT has been described before (15). The vector pMIEP(-1145/+112)CAT was constructed by replacing the *SacI*-*SmaI* fragment of pMIEP(-1145/+7)CAT with the above-mentioned *SacI*-*MaeIII* fragment. The plasmid pEXO1 was engineered by ligating complementary synthetic oligonucleotides, AGCTTGACCTCCATAGAAGACCC and GGGTGTCTTCTATGGAGGTCA, into the pUC19 vector between the *HindIII* and the *SmaI* sites. A similar strategy was used for the construction of the series of point mutations in the conserved box, resulting in plasmids pEXM1 through pEXM5. The specific oligonucleotides are shown in Fig. 7. The construction of the core promoter pMIEP(-66/+7)CAT was achieved by replacing the *EcoRI*-*SacI* fragment from pRR56/5 (kindly provided by B. Fleckenstein) with the *EcoRI*-*SacI* fragment from pMIEP(-1145/+7)CAT. The filled-in EXO1 oligonucleotide was cloned in multiple copies upstream of the core promoter by ligation into blunted *HindIII*-restricted pMIEP(-66/+7)CAT vector. The dimers of EXO1 in the sense and antisense orientations are termed p2CB(S)CAT and p2CB(AS)CAT, respectively. All cloning steps were confirmed by directly sequencing the plasmid DNAs and by restriction endonuclease cleavage.

Transfection assays. Transfections were performed by the calcium phosphate precipitation method on monolayers of HeLa S3 cells which were approximately 80% confluent. Cells were harvested and assayed for chloramphenicol acetyltransferase (CAT) activity as described by Gorman et al. (21). In each assay, 200 μ g of protein was assayed for CAT activity at 37°C for different time intervals between 5 and 25 min. The reaction consisted of a total volume of 150 μ l containing 0.47 M Tris-HCl (pH 7.8), 0.53 mM acetyl coenzyme A, 0.1 μ Ci of [¹⁴C]chloramphenicol (40 to 50 mCi/mmol), and cell extract. The products were extracted with ethyl acetate, separated by ascending thin-layer chromatography, and quantitated by scintillation counting.

Nuclear extract preparation. Nuclear extract from HeLa S3 cells was prepared as described by Dignam et al. (9), with the exception that all buffers contained 10 μ M each leupeptin, pepstatin, and aprotinin in addition to 0.5 mM phenylmethylsulfonyl fluoride (PMSF). Protein concentration was determined by the Bradford method (5).

In vitro transcription and competition assays. The transcription reaction conditions (in 25 μ l) were as described by Ghazal et al. (16). Routinely, poly(U) polymerase activity present in the extracts was used as an internal control to account for variability during the workup of the RNA samples. Experiments designed to dissociate the initiation and elongation steps were based on the studies of Hawley and Roeder (23). For these experiments, extracts were incubated for 20 min at 25°C before the addition of template DNA and ribonucleotides (50 μ M each CTP, GTP, and ATP and 10 μ M UTP). These modified conditions limit the ability of the transcription system to support more than one round of transcription (23). Preinitiation transcription complexes were assembled on pMIEP(-1145/+7)CAT and pMIEP(-1145/+112)CAT templates by incubating the DNA templates on ice for 10 min. A rapid start complex was initiated by incubating the preinitiation reaction at 30°C for 2 min. Ribonucleotides, described above, were added, rapidly followed (within 30 s) by the addition at room temperature of

Sarkosyl as indicated in the legend to Fig. 3. Elongation reactions were allowed to proceed for 15 min at 30°C and processed as described below.

The in vitro transcription oligonucleotide competition assay was performed as described previously (18). The human immunodeficiency virus (HIV) long terminal repeat (LTR) template pBennCAT was truncated at the *EcoRI* site downstream from the initiation site of transcription and used in the in vitro transcription system as described before (34). In vitro transcription oligonucleotide competition experiments with the templates pMIEP(-1156/+112)CAT and pBennCAT were performed independently. The RNA products synthesized from these experiments were pooled and coelectrophoresed. All transcription reactions were allowed to proceed for 15 min, after which the in vitro-synthesized RNA was processed by stopping the reaction with 100 μ l of 1% Sarkosyl-10 mM Tris-HCl (pH 8.0)-0.2 M NaCl-40 μ g of tRNA per ml. The samples were phenol extracted once, followed by an ether extraction, and then ethanol precipitated. The pelleted nucleic acids were resuspended in 8 μ l of 90% formamide-1 \times gel buffer (0.1 M Tris-borate, 2 mM EDTA [pH 8.3])-0.1% bromophenol blue-0.01% xylene cyanol and heated to 90°C for 5 min. Resuspended RNA transcripts (4 μ l) were electrophoretically separated on a 4% sequencing gel and visualized by exposure of the dried gels to Kodak X-Omat AR film with an intensifying screen at -70°C. For quantitation, the run-off transcripts were excised from the dried gel, and the amount of Cerenkov radiation per specific transcript band was determined.

DNase I protection analysis. The exon 1 fragment for DNase I protection studies was prepared from the pUCExon1 vector containing the MIEP sequences from -19 to +112. Endonuclease cleavage at the *HindIII* and *EcoRI* sites of pUCExon1 was used to release the fragment from the vector. The *HindIII* and *EcoRI* sites were selectively dephosphorylated by treatment with alkaline phosphatase. The fragments were subsequently gel purified and labeled with polynucleotide kinase and [γ -³²P]ATP. The conditions for the DNase I protection experiments were as described previously (16).

Gel mobility shift assay. The gel mobility shift assay and binding reaction conditions were performed as described before (17). The cloned double-stranded oligonucleotides from pEXO1, pEXM1, pEXM2, pEXM3, pEXM4, and pEXM5 were released from the vector with the endonucleases *HindIII* and *SmaI* and then gel purified. The double-stranded oligonucleotide probes were end-labeled with [α -³²P]dATP by using Sequenase version 2.0 (USB). Approximately 0.1 to 0.5 ng of radiolabeled probe was used per binding reaction.

Photoactivated DNA-protein cross-linking. Photoactivated DNA-protein cross-linking in solution was performed by specifically binding the radiolabeled EXO1 fragment (0.5 ng) with 20 μ g of crude nuclear extract and irradiating the complex with 80,000 μ J by using UV Stratalinker 1800. In situ photoactivated DNA-protein cross-linking was achieved by resolving the above specific complexes in a 2% low-melting-point agarose gel mobility shift, irradiating the gel with 240,000 μ J. The nucleoprotein complex was visualized by autoradiography, and the bound and free DNA were excised, boiled for 5 min in the presence of 1% sodium dodecyl sulfate (SDS) and β -mercaptoethanol, and directly loaded on an 8% Laemmli gel. ¹⁴C-methylated protein markers (myosin [200 kDa], phosphorylase b [100 and 92.5 kDa], bovine serum albumin [69 kDa], and ovalbumin [46 kDa]) were purchased from Amersham.

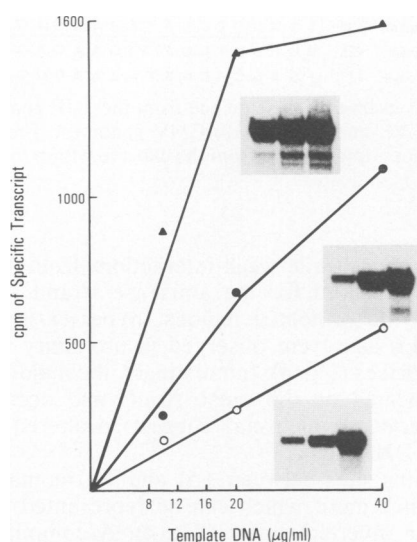


FIG. 1. In vitro transcription run-off assays with different concentrations of DNA templates pMIEP(-1145/+7)CAT (○), pMIEP(-1145/+54)CAT (●), and pMIEP(-1145/+112)CAT (▲) truncated with *Eco*RI, *Pvu*II, and *Pvu*II, respectively. The transcription reactions were performed, processed, and subjected to polyacrylamide gel electrophoresis as described under Materials and Methods. The specific run-off transcripts were excised from the gel, and the amount of transcript was determined as described under Materials and Methods.

RESULTS

Exon 1 sequences contain a transcription control domain.

The importance of DNA sequences that lie downstream from the start site of transcription in controlling the level of expression from MIEP is not known. Since this region may contain a unique target for regulating viral gene expression, we sought to explore the involvement of downstream sequences in regulating MIEP activity by using an RNA polymerase II in vitro transcription system. The de novo synthesis of RNA in the in vitro assay permits one to elucidate the requirement of this control domain in transcription. The DNA templates used in this assay comprised deletion mutations in the 5' untranslated leader exon (exon 1) fused to the reporter gene for CAT (*cat*). The plasmids pMIEP(-1145/+7)CAT, pMIEP(-1145/+54)CAT, and pMIEP(-1145/+112)CAT, containing MIE leader exon 1 sequences deleted at +7, +54, and +112, respectively, were assayed in HeLa S3 transcriptionally active nuclear extracts.

In order to obtain similar-length transcripts, pMIEP(-1145/+7)CAT was resected with *Eco*RI, while pMIEP(-1145/+54)CAT and pMIEP(-1145/+112)CAT were resected at the *Pvu*II site, resulting in run-off transcripts truncated 263, 220, and 265 nucleotides downstream of the initiation site, respectively. A significant enhancement of transcription was observed in those constructs containing exonic sequences (Fig. 1). The transcription enhancement for pMIEP(-1145/+54)CAT relative to pMIEP(-1145/+7)CAT was threefold and for pMIEP(-1145/+112)CAT relative to pMIEP(-1145/+54)CAT was twofold (Fig. 1). Therefore, the enhanced level of transcription in vitro mediated by the complete exon 1 sequences was approximately sixfold.

In support of the in vitro run-off experiments, in vivo transfection assays with the deletion mutant templates also demonstrated an augmentation of MIEP activity by as much

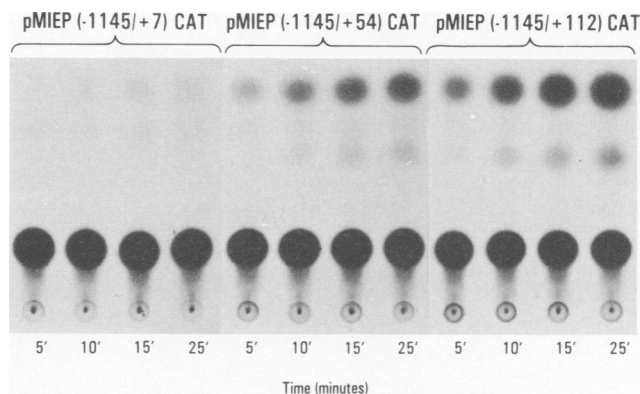


FIG. 2. Kinetic analysis (from 5 to 25 min) of CAT enzyme activity from deletion mutant constructs pMIEP(-1145/+7)CAT, pMIEP(-1145/+54)CAT, and pMIEP(-1145/+112)CAT containing 3'-end deletion endpoints at +7, +54, and +112, respectively. These *cat* constructs were transiently transfected into HeLa S3 cells; extracts were prepared 48 h later, and CAT activity was determined by thin-layer chromatography.

as 15- to 18-fold (Fig. 2). The deletion mutations suggest that the 5' untranslated leader exon of the MIE gene may interact with nuclear factors to promote the process of transcription complex formation and elongation. The additive stimulatory effect seen with increasing amounts of the leader sequences suggested the involvement of multiple elements.

Leader control domain enhances the rate of transcription initiation by RNA polymerase II. The conditions used for the in vitro transcription were not completely limiting for a single-round transcription (see Materials and Methods), and reinitiation events could still have occurred (Fig. 1). If the elongation rate is enhanced by the exonic leader sequences, then presumably RNA polymerase II molecules can begin a new round of initiation sooner. Consequently, under these conditions, the observed effect on the number of initiations could be indirect. To determine whether transcription complexes which formed on the different templates were primarily initiation or elongation complexes, the effect of the anionic detergent Sarkosyl on specific transcription was examined. Elongation of committed transcription complexes is insensitive to the presence of Sarkosyl (0.02%), whereas transcription complexes formed prior to elongation are disrupted (see Materials and Methods). Therefore, the relative rates of initiation of transcription can be dissociated from the rates of transcription elongation. Under these defined conditions for a single round of transcription in the in vitro transcription assay, pMIEP(-1145/+112)CAT gave an approximately four- to fivefold increase in the level of RNA synthesis compared with pMIEP(-1145/+7)CAT (Fig. 3). Furthermore, a comparison of the level of transcription for this experiment in the presence and absence of Sarkosyl demonstrated that reinitiation did not occur. We therefore conclude that the enhanced level of transcription, mediated by the leader exon sequences, is due to the rate of initiation complex formation.

Binding of nuclear proteins to multiple sequence motifs in exon 1. To explore whether sequence-specific factors bind to defined exonic leader elements, DNase I protection experiments were performed with the transcriptionally active extract. Strong protection was observed with both the sense and antisense strand sequences between +53 and +78 (Fig. 4). Weaker interactions were detected between +34 and +54

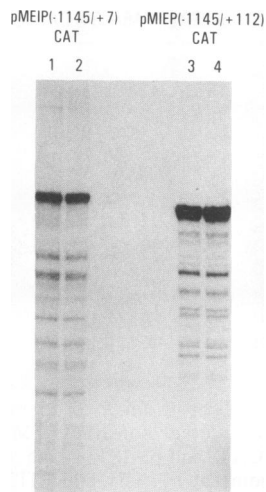


FIG. 3. Single-round RNA chain initiation from pMIEP(-1145/+7)CAT and pMIEP(-1145/+112)CAT. These DNA templates were restricted with *EcoRI* and *PvuII*, respectively, and used in the *in vitro* transcription assay under limiting conditions for reinitiation as detailed in Materials and Methods. In lanes 2 and 4, Sarkosyl was added to a final concentration of 0.02% 30 s before nucleotide additions. Water instead of Sarkosyl was added in lanes 1 and 3. Quantitation of specific transcript was achieved by Cerenkov counting of the excised transcript band from the dried gel.

Human	+33	T G A C C T C C A T A G A A G A C A
Simian	+25	G G A G C T C C A T T G G A A G A G A
Mouse	+17	T G A C C A C C G T A G A A C G C A

FIG. 5. Conserved box sequence from the MIE genes of human (3), simian (28), and murine (10) CMV genomes. The location of these sequences downstream from the cap site within the 5' untranslated exon 1 is indicated.

for both strands, while weak interactions from +79 to +91 were only observed for the antisense strand (Fig. 4). In addition to the protected regions, hypersensitive sites of DNase I cleavage were observed in proximity to the protected sequences (Fig. 4). Interestingly, the majority of these sites are located on the sense strand and occur at fairly regular intervals, which may suggest an altered conformation of the DNA helix.

The binding sites between +51 and +91 contain a reiterated sequence motif which can be represented as either a direct or an inverted repeat (Fig. 4). A computer-assisted search revealed a conserved sequence element associated with the +34 to +54 binding site (Fig. 5). All of the downstream elements required for MIEP transcriptional activity map within the sites of protein-DNA interaction. We cannot rule out that additional sequences undetected in this study may be involved. However, a good correlation is observed between the binding sites for nuclear protein(s) and the transcriptional activity of the deletion mutations. This strongly supports the conclusion that the binding of these sequence-specific factors is responsible for the increased rate of initiation of transcription.

Recognition of a conserved sequence element by discrete cellular factors. The conserved box-binding site between +34 and +54 was only weakly protected in the DNase I protection experiments (Fig. 4). It is possible that this binding activity represents an extension of the strong downstream footprint or that the conserved box may bind an independent factor(s). The latter suggestion is supported by

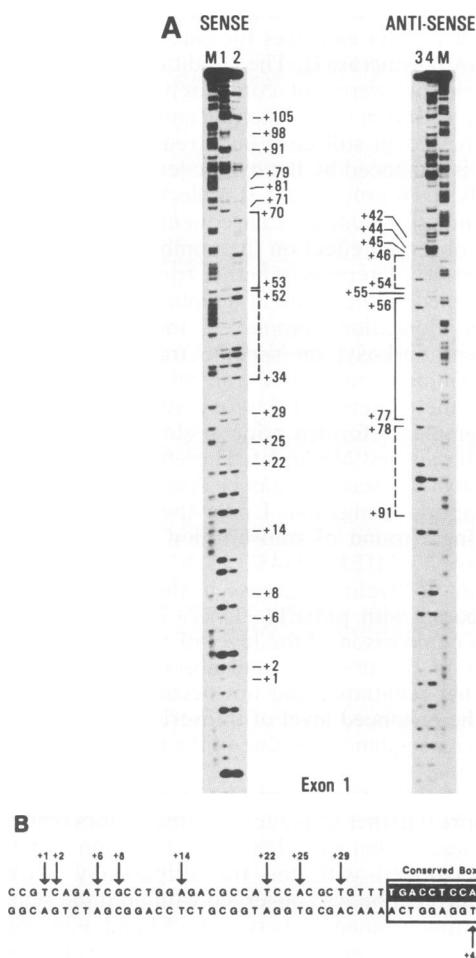


FIG. 4. (A) DNase I footprinting of cellular proteins interacting with the 5' untranslated leader exon of MIEP. HeLa S3 nuclear proteins were analyzed for sequence-specific DNA-binding proteins interacting with exon 1 sense and antisense strands by DNase I footprinting as described under Materials and Methods. Lanes 1 and 4 demonstrate the DNase I digestion pattern in the presence of nuclear proteins. Lanes 2 and 3 demonstrate a control reaction containing DNase I alone. Lane M is an A+G Maxam and Gilbert sequence ladder of the exon 1 fragment. Brackets mark regions of DNase I protection, with the location indicated by the number of base pairs downstream from the cap site. Horizontal lines indicate DNase I-hypersensitive sites that were reproducibly observed. (B) Sites of protein-DNA interaction on the MIEP leader exon region. Numbers represent the distance in base pairs downstream from the transcription start site (+1). Sequences protected from DNase I cleavage are marked by white letters on a black background. Vertical arrows denote positions of enhanced protein-induced DNase I cleavage *in vitro*. Repeated sequence elements are designated by horizontal arrows, and the conserved sequence element is boxed.

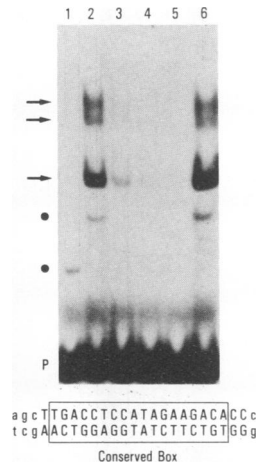


FIG. 6. Nucleoprotein complexes associated with the conserved box in the gel mobility shift assay. Nuclear extract from HeLa S3 cells was incubated with the EXO1 oligonucleotide probe indicated by the sequence. Capital letters of the sequence mark nucleotides within the exon 1, while lowercase letters refer to linker sequences. P marks the location of free probe. Lane 1, Probe in the absence of specific nuclear proteins; lanes 2 to 6, probe in the presence of 5 μ g of HeLa cell nuclear proteins; lanes 3 to 6, the binding reactions contained a 50-fold molar excess of competitor DNAs. The competitor DNA fragments were (lane 3) unlabeled EXO1, (lane 4) MIEP fragment -19 to +54, (lane 5) MIEP fragment -19 to +112, and (lane 6) MIEP fragment -19 to +7. Arrows indicate specific nucleoprotein complexes.

the observation that the deletion mutant pMIEP(-1145/+54)CAT retains stimulatory activity. To examine in more detail whether the conserved box can independently bind specific nuclear proteins, a mobility shift assay was performed. The assay used a synthetic double-stranded oligonucleotide (EXO1) corresponding to the conserved box. Figure 6 clearly shows that the conserved box can independently interact with nuclear proteins and supports the DNase I protection experiment results.

In this experiment, a complex pattern is observed, suggesting that multiple nucleoprotein complexes may be associated with the conserved element. Competition experiments were used to establish the sequence specificity of these complexes. Three predominant complexes (indicated by the arrows in Fig. 6) were inhibited from forming by an excess of the unlabeled EXO1 oligonucleotide, a MIEP fragment containing sequences between -19 and +54 and the exon 1 fragment containing sequences from -19 to +112. However, a fragment containing sequences from -19 to +7 did not inhibit the formation of the three specific nucleoprotein complexes (Fig. 6, lane 6). The complex indicated by the dot represents a single-stranded component of the oligonucleotide that is specifically band shifted but was not reproducibly observed. The two slower-migrating complexes were occasionally resolved as a single band and are designated together as complex A (Fig. 7). We refer to the predominant fast-migrating band as complex B. Furthermore, the cell type distribution of the conserved box complexes A and B appears to be constitutive in a wide variety of cells, including Jurkat, CEM, U937, H9, Raji, B, HepG2, human foreskin fibroblasts, NT2-D1, and T47D cells (data not shown). However, the functional activity of this element and its cognate binding factors in these respective cell types remains to be determined.

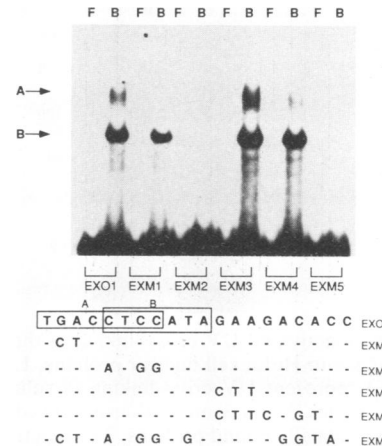


FIG. 7. Binding of nuclear proteins to the mutated conserved box templates. The sequence of the wild-type motif (EXO1) is shown below the figure, and the point mutations present in each of the respective mutant templates are indicated immediately below. The name of the template in each line is indicated. The locations of the A and B complexes formed with the wild-type and mutant templates are indicated on the left-hand side and boxed in the EXO1 sequence. F represents free probe in the absence of nuclear proteins, while B represents bound probe in the presence of nuclear proteins.

The sequence requirement for the formation of the conserved box complexes was investigated further in the gel retardation experiments with a series of radiolabeled templates containing point mutations throughout the conserved box motif (Fig. 7). Formation of complex A (Fig. 7) was completely inhibited by point mutations at positions +34, +35, +37, +39, and +40 (mutations EXM1 and EXM2). These observations indicate that complex A recognizes the TGACCTCC motif of the conserved box. In contrast, complex B was completely inhibited by point mutations at positions +37, +39, and +40 (EXM2), while mutations at +44, +45, +46, +47, +49, and +50 had little effect (EXM3 and EXM4). We conclude from these observations that complex B recognizes sequences between +37 and +43, representing the CCTCCATA motif. Mutations at +44, +45, and +46 (EXM3) slightly increased the formation of complexes A and B, while additional mutations at positions +47, +49, and +50 slightly decreased complex A formation. The observation that sequence outside the recognition motif influences the extent of binding indicates that the sequence context of complex A and B binding may be important. Alternatively, a direct interaction may exist for complex A and B with sequences located between positions +44 and +50. Importantly, complex A was exclusively inhibited from forming by mutations at +34 and +35 (EXM1), while complex B was unaffected, strongly suggesting different proteins in the formation of complex A and B that appear to interact with closely overlapping sequences. The complexes A and B formed on the mutated templates could be inhibited by the wild-type (EXO1) template, demonstrating that no new complex was formed (data not shown).

The discrete nature of complexes A and B was further supported by photoactivated UV cross-linking experiments in solution, which identified two distinct proteins of 50 ± 4 and 74 ± 4 kDa, respectively, that recognized the conserved box (Fig. 8). Competitor fragments containing the conserved box-binding site specifically competed for the 74- and 50-kDa

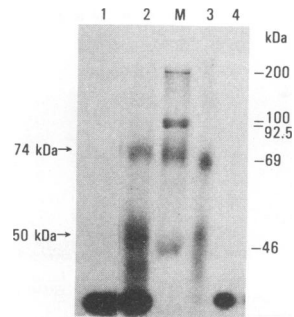


FIG. 8. Photoactivated UV cross-linking of the conserved box-binding sequence to HeLa cell nuclear proteins. Lanes 1 and 2 and lanes 3 and 4 represent UV cross-linking in solution and in situ, respectively. Lanes 2 and 3 are UV-cross-linked products in the presence of specifically bound proteins. The reaction conditions for cross-linking are detailed in Materials and Methods. M, Marker proteins. Specific polypeptides (arrows) in this assay are shown by their determined molecular masses of 74 ± 4 and 50 ± 4 kDa.

proteins in the solution cross-linking experiment (data not shown). The amount of label in these species increased with increasing UV irradiation, with the optimal irradiation detected between 80,000 and 100,000 μ J (data not shown). Labeled species were not observed when the probe DNA alone was irradiated (Fig. 8) or when UV irradiation was omitted (data not shown).

An alternative strategy was also used to identify the molecular weights of the proteins bound to the conserved box. In this experiment, the double-stranded EXO1 oligonucleotide was bound with nuclear proteins, and the specific nucleoprotein complexes were then resolved in a 2% agarose gel. In the agarose gel, only a single nucleoprotein complex was developed with the EXO1 probe that could be specifically competed with by unlabeled EXO1 (data not shown). The resolved complex was irradiated in situ, excised, and electrophoretically separated on an SDS-polyacrylamide gel (Fig. 8). Two specific complexes which comigrated at approximately the distance of the specific products from the solution UV cross-linking experiment were detected (Fig. 8). Since the covalent attachment of short oligonucleotides to proteins has only a minor effect on the mobility of these proteins in SDS-polyacrylamide gels, these experiments indicate that the molecular weights of the proteins identified above are close to the apparent molecular weights predicted by the gels.

Conserved sequence box functions at the DNA level. To investigate whether the conserved box is recognized at the DNA level, the EXO1 oligonucleotide was dimerized upstream of the core promoter (see Materials and Methods). Run-off in vitro transcription reactions were performed with pMIEP(-66/+7)CAT, p2CB(S)CAT, and p2CB(AS)CAT (Fig. 9). To control internally for variability during processing of samples, poly(U) polymerase activity present within the nuclear extracts was assayed simultaneously during the transcription reactions (Fig. 9). Transcripts were quantitated relative to the poly(U) polymerase RNAs by scintillation counting of bands. A 2.5- and 1.8-fold stimulation in transcription was observed for p2CB(S)CAT and p2CB(AS)CAT relative to pMIEP(-66/+7)CAT, respectively (Fig. 9). A moderate increase was also observed with the monomers, although there was a strong requirement for multiple sequence elements for optimal activity. We conclude that the



FIG. 9. Oligomerization of conserved box (EXO1) upstream of the core promoter, pMIEP(-66/+7)CAT. Dimers of EXO1 in the sense and antisense orientation are present within the constructs of p2CB(S)CAT and p2CB(AS)CAT, respectively. Run-off in vitro transcription assays were performed with *Eco*RI-truncated templates of: lane 1, pMIEP(-66/+7)CAT; lane 2, p2CB(AS)CAT; and lane 3, p2CB(S)CAT. The specific run-off transcripts are indicated by the IS arrow, and the internal control [poly(U) polymerase-labeled RNA] are indicated by the IC arrow. Quantitation of specific transcription was achieved by cutting out specific transcript bands and Cerenkov counts were normalized to the respective IC-labeled bands.

conserved box can act independently upstream of the promoter and thus is recognized at the DNA level.

To examine further whether the binding of the factor(s) to the DNA sequence of the conserved box plays a role in activating MIEP, we performed in vitro transcription oligonucleotide competition experiments with the duplex deoxy-oligonucleotide, EXO1. Increasing molar amounts of EXO1 relative to the concentration of pMIEP(-1145/+112)CAT inhibited the specific level of transcription approximately threefold (Fig. 10). A nonspecific competitor oligonucleotide did not inhibit the level of transcription (data not shown). In addition, equivalent molar amounts of EXO1 did not affect specific transcription from the HIV-1 LTR (Fig. 10). We conclude from these experiments that the conserved box sequence is recognized at the DNA level by a sequence-specific transcription factor(s) necessary for the activation of MIEP. An examination of the effect of the mutant duplex oligonucleotides in the in vitro transcription competition assay was performed. Those mutants which were bound by both complexes A and B competed efficiently (EXM3 and EXM4), while those that bound neither (EXM2 and EXM5) or only one (complex EXM1) failed to compete significantly (Fig. 11). Thus, a direct correlation is observed between binding of complexes and function of the conserved box. In particular, these data clearly demonstrate the requirement for complex A in the activation of MIEP.

DISCUSSION

Leader transcription control domain of MIEP. In this study we have identified a positive transcriptional control domain within the 5' untranslated leader region, exon 1, of the

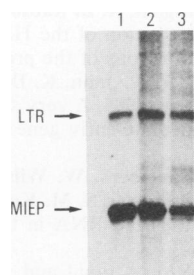


FIG. 10. In vitro transcription oligonucleotide competition assay with EXO1 oligonucleotide and HIV LTR (pBennCAT) and pMIEP (–1145/+112)CAT templates truncated at the *Eco*RI and *Pvu*II sites, respectively. Reactions were performed as described in Materials and Methods. Arrows mark the specific transcripts from pBennCAT (LTR) and from pMIEP(–1145/+112)CAT (MIEP). Lane 1, Transcription reactions in the absence of competitor DNA; lanes 2 and 3, reactions in the presence of EXO1 competitor at 20- and 50-fold molar excess over the template DNA, respectively.

HCMV IE UL123-122 gene. This promoter-proximal downstream domain functions by increasing the number of committed RNA polymerase II transcription complexes. In the presence of a very strong enhancer, this leader region can contribute significantly to the level of expression from MIEP by as much as 18-fold and 6-fold in vivo and in vitro, respectively.

The activity of the leader transcription control domain is mediated by sequence-specific DNA-binding proteins which we designate LTFs (leader transcription factors). The binding of these factors detected in this study occurs with sequences between +33 and +91. The sequences bound by specific DNA-binding proteins between +33 and +51 represent a sequence element that occurs in approximately the same location downstream from the MIEP cap sites in strains of CMV from different species (Fig. 5). This conserved element interacts with two discrete nucleoprotein

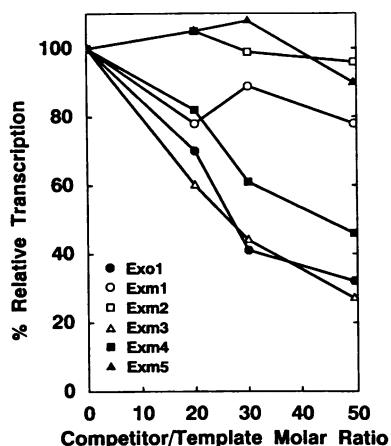


FIG. 11. Effect of the different mutant conserved box oligonucleotides on the transcriptional activity of pMIEP(–1145/+112)CAT in an in vitro competition assay. The amount of competitor is expressed as a molar ratio with respect to the template. Quantitation was achieved by scintillation counting of transcripts and normalized to the HIV LTR transcripts. The relative transcriptional activity of a template is plotted as a function of increasing competitor/template molar ratio and expressed as a percentage of its transcriptional level in the absence of competition.

complexes, designated LTF A and B, that contact closely overlapping sequences. Here we present evidence for the requirement of LTF A activity in regulating MIEP from oligonucleotide in vitro transcription competition experiments with the mutant conserved box templates.

The conservation of a sequence motif in approximately the same position within the 5' untranslated leader exon 1 of the CMV MIEPs suggests an important biological function of these sequences. This homology is particularly striking in the case of murine CMV, in which the conserved box represents the only significant identity within the complete exon 1 sequences (10). A more extensive homology is detected between the human and simian exon 1 sequences, which begins at the conserved box and extends to the repeat motifs between nucleotides +52 and +91 (28). The oligonucleotide competition experiments and oligomerization upstream of the promoter, in combination with the deletion mutation analysis, strongly support the conclusion that the conserved box plays an important role in MIEP transcriptional activity. Although we have in this study focused on the conserved box, we anticipate that additional elements within exon 1 may be involved in regulating MIEP transcription activity, which will be the subject of future investigation. It is also possible that exon 1 sequences contain elements which control posttranscriptional events such as the stability of the RNA molecule. Alternatively, LTFs may be involved in negating repression of transcription mediated by nucleosomal cores and histone H1. We propose, therefore, that the leader domain may contribute a key target for regulating viral activation in a permissive cell. It is important to note that although HeLa cells are nonpermissive for HCMV growth, the permissive human foreskin fibroblast cells contain LTF activity (unpublished observations). To demonstrate the functional activity of LTFs in human foreskin fibroblast cells, it will be necessary to establish a reconstituted in vitro transcription system from these cells. In this connection, it will ultimately be necessary to target this regulatory domain for mutagenesis in the viral genome in order to assess its importance during the viral replication cycle.

At present, it is unclear whether the conserved box-binding factors occur downstream of promoters for cellular genes. The ability of the conserved box to function upstream of the MIEP promoter strongly suggests that its position downstream may not be a requirement in cellular promoters. However, LTF binding sites between +52 and +91 of the MIEP bear some resemblance (approximately 70% identity) to sequences located shortly downstream of the initiation sites for the mouse dihydrofolate reductase (11) and rpl32 (22) genes. These cellular sequences are in a region required for promoter activity and also have been shown to bind cellular proteins (11, 22).

Interactions with the transcriptional machinery. We conclude that the leader transcription control domain is distinct from the core promoter, since correctly initiated complexes are detected in the absence of the leader region. In this respect the leader control domain is distinct from the internal control domains of RNA polymerase I and III genes.

In general, promoter-proximal downstream transcriptional elements may be classed as elements which are recognized at either the RNA level or the DNA level. Within these classes, two mechanistic distinctions can be made which include those elements which affect initiation and those that affect translocation of the transcription complex. In some cases, a particular downstream promoter-proximal region may encompass all of the above characteristics, as is exem-

plified by the intensely studied HIV LTR (2, 4, 12–14, 27, 29–31, 36, 42–45, 47, 48, 50, 55). An increasing number of RNA polymerase II promoters have been shown to contain essential DNA sequences located downstream of the initiation site of transcription, although little is known about the mechanism of action (1, 8, 11, 13, 22, 25, 29, 32, 35, 40, 53, 56, 57). However, in the case of the simian virus 40 late promoter, it has been shown that the downstream element increases the number of functional preinitiation complexes assembled at the promoter without altering the apparent rate of complex assembly (1). It was suggested that in the absence of the downstream DNA element, preinitiation complexes were partially assembled but were not transcriptionally competent. In the study presented here on the MIEP, we have described the activity of a transcriptional downstream domain that appears to be mediated by a sequence-specific DNA-binding protein(s) that affects the rate of initiation complex formation. Although we have not measured the rate of complex assembly, we have shown that the downstream domain enhances the number of competent initiation complexes. These observations raise a number of interesting possibilities by which the transcription factors may interact with the general transcription machinery. For example, the binding of LTFs to exon 1 may function in the stabilization or recruitment of the initiated promoter complex. In this example, such interactions may exist transiently by interacting with the preinitiation complex, disengaging upon committed complex formation, and recycling to the next preinitiation complex. Alternatively, the LTFs may be engaged with both the preinitiation and committed transcription complexes. More interestingly, LTFs may function by a novel mechanism of action involving a quaternary complex interaction (DNA-RNA-LTF-transcription complex) as opposed to a ternary complex (DNA-LTF-transcription complex). These different possibilities remain speculative and must await further purification and isolation of the LTFs and their analyses in the reconstituted *in vitro* system.

In conclusion, we have described the first example of a transcription control domain, outside overlapping transcription units, internal to a CMV gene in which the viral genome length is approximately 240 kb. We propose that the leader control domain of MIEP may constitute a key target for regulating viral activation. This downstream control domain functions by increasing the number of initiation complexes at the promoter and involves the interaction of at least two novel cellular sequence-specific factors. The binding of these factors to their target DNA does not appear to impede the apparent rate of elongation by RNA polymerase II. Finally, this transcription control region underscores the diversity of control domains associated with mammalian RNA polymerase II transcription units.

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